

Encapsulation of bacteria and viruses in electrospun nanofibres

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Abstract

Bacteria and viruses were encapsulated in electrospun polymer nanofibres. The bacteria and viruses were suspended in a solution of poly(vinyl alcohol) (PVA) in water and subjected to an electrostatic field of the order of 1 kV cm^{-1} . Encapsulated bacteria in this work, (*Escherichia coli*, *Staphylococcus albus*) and bacterial viruses (T7, T4, λ) managed to survive the electrospinning process while maintaining their viability at fairly high levels. Subsequently the bacteria and viruses remain viable during three months at -20 and -55°C without a further decrease in number. The present results demonstrate the potential of the electrospinning process for the encapsulation and immobilization of living biological material.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

The encapsulation of biological material in a dry form while preserving its activity is important for many applications. An extension of this is the encapsulation of complete organisms. For example, there has recently been a greatly increased interest in using bacterial viruses as an alternative to bacterial antibiotics (phage therapy) and as vectors for gene delivery (viral and non-viral vectors) [1–3]. These uses require the development of means for efficient encapsulation that ensure that such bacterial viruses can be delivered to a desired destination both intact and viable. The present work aims to investigate electrospinning as a possible method of encapsulating both bacteria and bacterial viruses. Our aim was to elucidate the conditions of the electrospinning process that allow the encapsulation of intact bacteria and bacterial viruses while maintaining their viability. In addition, the conditions for storing such material were examined.

Electrospinning is a common method to produce nanofibres with a diameter in the range of 100 nm or even less [4–6]. In this process a polymer solution is supplied from a spinneret and forms a droplet at the spinneret exit. In the presence of an electrical field applied to the solution by immersing an electrode in it and placing a counter-electrode some distance from the spinneret, the Maxwell electrical stress stretches the droplet, a Taylor cone is made and jetting

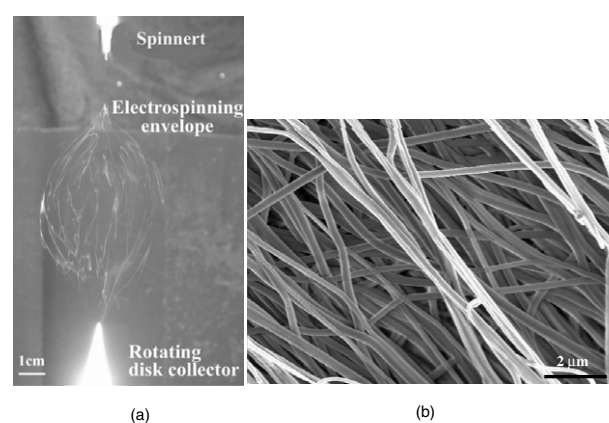


Figure 1. A photograph of the spinning process between the spinneret and the rotating disc collector, taken at a low shutter speed (200 ms), (b) an HRSEM micrograph of a mat formed by electrospun PVA nanofibres.

sets in [7]. The jet exhibits an electrically-induced bending instability which causes stretching of the bent sections of the jet [8–10]. The solvent eventually evaporates, the jet dries and solidifies, and the as-spun nanofibres are deposited on the counter electrode. A typical electrospinning setup is presented in figure 1(a) (cf [11]). In this setup, the polymer solution

is supplied from a syringe and the electrospun nanofibres are collected (figure 1(b)) on a sharpened collector disc which acts as an electrostatic lens that pulls the spun nanofibres to a focal point, namely the edge of the disc.

Two characteristic features of the electrospinning process are: (1) the extremely rapid formation of the nanofibre structure, which is on a millisecond scale, and (2) the huge material elongation rate of the order of 1000 s^{-1} , which is accompanied by a cross-sectional area reduction of the order of 10^5 [7, 8, 12]. A typical electrical Bond number for the electrospinning process, close to the droplet, is of the order of $B_{OE} = r \cdot E_\infty^2 / \gamma = 10$, where E_∞ is the electric field strength and of the order of 1 kV cm^{-1} (assuming a uniform electrostatic field), the jet radius r is about 10^{-3} cm , and the surface-tension coefficient γ of the polymer solution is about 50 g s^{-2} .

The feasibility of incorporating particles into electrospun nanofibres has been demonstrated in recent work [13–15]. Here we use the electrospinning technique to embed both spherical and rod-like bacteria and bacterial viruses in a polymer matrix, which forms a composite nanofibre during electrospinning. The individual bacteria can be discerned within these fibres. The bacteria or viruses are initially dispersed in a polymer solution and randomly oriented. Due to the sink-like flow at the Taylor cone, the rod-like bacteria and viruses are gradually oriented, mainly along the stream lines, so that aligned organisms are pulled into the jet in an almost oriented manner [13]. A schematic illustration of rod-like particles at the sink-like flow through a Taylor cone is presented in figure 2. The tangential stress, σ_τ , and the normal stress, σ_n , applied to the particles at this stage are of the order of $5 \times 10^3 \text{ g cm}^{-1} \text{ s}^{-2}$ [12]. Additional radial pressure in the as-spun nanofibres ensues due to surface tension resulting in contraction of the polymer matrix. The pressure caused by surface tension forces in the case of a cylindrical fibre is $p = \gamma/r = 5 \times 10^4 \text{ g cm}^{-1} \text{ s}^{-2}$.

Biological material has previously been encapsulated in electrospun fibres. For example, DNA has been encapsulated for potential therapeutic applications in gene therapy [16]. It was found that plasmid DNA released directly from the electrospun scaffold was indeed intact, capable of transforming cells, and still encoded the alpha portion of the enzyme β -galactosidase. Filamentous bacterial viruses suspended in a polymer solution were electrospun and found to remain viable when examined immediately after electrospinning [17]. However, no data were presented as to the numbers of infective particles before and after spinning and whether infectivity is preserved in this spun material. This is obviously important for the use of such material for phage therapy. Some proteins, enzymes and small molecules have also been embedded in electrospun nanofibres [18–22].

In this work we demonstrate the feasibility of encapsulating whole organisms, both viruses (T7, T4, λ) and bacteria (*Escherichia coli*, *Staphylococcus albus*), in poly(vinyl alcohol) (PVA) electrospun nanofibres. Furthermore, it is shown herein that these organisms remain viable. In addition, we investigated whether such fibres might be an efficient and facile method to preserve these organisms and it was found that once embedded, such organisms retain full viability for 3 months when held at both -20 and -55°C , and nearly so at 4°C .

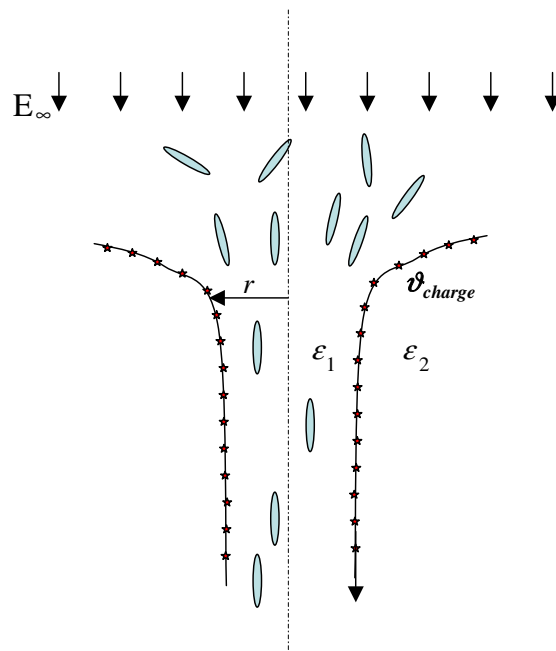


Figure 2. Schematic illustration of rod-like bacteria at the sink-like flow. The bacteria are initially dispersed in a polymer solution and randomly oriented. Due to the sink-like flow, the rod-like bacteria are gradually oriented mainly along the stream lines. The mechanical stresses on the bacteria are σ_τ and σ_n . Where E_∞ is the uniform electric field applied to the droplet, v_{charge} is the charge density of those charges located on the fibre envelope, ϵ_1 and ϵ_2 are the electrical permittivity of the polymer solution and the ambient gas, respectively. r is the jet diameter.

2. Materials and methods

2.1. Biological materials

The bacteria examined in this work were *Escherichia coli* and *Staphylococcus albus*. *E. coli* are rod-shaped bacilli with a diameter of about $1 \mu\text{m}$ and an average length that is twice that. *E. coli* is easy to grow and count, both microscopically and biologically. The prototrophic strain used was W3110 which is a type K12 strain. It can be grown in a defined medium (Vogel–Bonner medium E) [23] of inorganic salts with glucose as the carbon source. Batch cultures grown in this growth medium at 37°C attain a density of about $1\text{--}2 \times 10^9 \text{ cells ml}^{-1}$. *S. albus* is a spherical bacterium (coccus) whose diameter is about 600 nm . The strain examined synthesizes the restriction endonuclease SalI^3 . This strain failed to grow in a defined medium but can be propagated in Luria–Bertani (LB) medium [24] which is a rich medium. A density of about $8 \times 10^9 \text{ cells ml}^{-1}$ can be reached in stationary phase cultures. When necessary, both bacterial cultures were centrifuged, washed with the Vogel–Bonner salts cited above without glucose and then suspended at the same density in a dilute salts solution. The cells remain highly viable for several weeks. The viruses (bacteriophages) examined in this work were T7, T4 and λ . All grow on the above strain of *E. coli* K12 and lysates of them were prepared on this strain in

³ The strain was generously provided by Dr C Yanofsky of the Department of Biological Sciences, Stanford University, Stanford, California.

LB medium. T4 is an intensively studied [25] bacteriophage whose icosahedral head (capsid) has a length and width of 110 and 85 nm, respectively, and a tail of about 130 nm length which is connected to six tail fibres which are the organelles of attachment to its bacterial host. The bacteriophage λ [26] is another well known *E. coli* virus that is often used for cloning relatively large pieces of DNA. It has a tail but only a single short tail fibre and a capsid whose diameter is about 50 nm. T7 is similar to λ but has a very short tail [27]. All three viruses can be titred and prepared in large amounts with great ease. More than 2×10^{13} viral particles can be prepared from a litre of infected bacteria.

2.2. Electrospinning

Electrospinning was carried out by making a suspension of bacteria or viruses in the polymer solution. The bacteria or the virus were dispersed in the dilute salt solution or LB media. This was mixed with an equal volume of 14% w/w aqueous solution of poly(vinyl alcohol) (PVA, MW = 10^5 g mol⁻¹, Aldrich), (see [28] for electrical and rheological characterization). The suspension underwent electrospinning from a 1 ml syringe with a hypodermic needle with an inner diameter of 0.5 mm. The flow rate was 0.2–0.5 ml h⁻¹. A copper electrode was placed in the polymer solution and the suspension was spun onto the edge of a grounded collector disc (for more details cf [11]). The strength of the electrostatic field was 1.1 kV cm⁻¹ and the distance between the electrode tip and the edge of the disc was 12 cm. The linear speed at the edge of the disc collector was $V = 8.8$ m s⁻¹. All the experiments were performed at room temperature ($\sim 24^\circ\text{C}$), and a humidity of about 50%.

2.3. Sample preparation

As-spun nanofibres were collected on the disc edge for 1 h and formed a macroscopic ribbon with well-aligned nanofibres. The ribbon was detached from the disc, cut into samples and distributed into four Eppendorf centrifuge tubes. A small sample was taken from one of these for viability tests and then the vials were stored at four different temperatures (room temperature ($\sim 24^\circ\text{C}$), 4°C , -20°C , -55°C). The as-spun fibres were found to contain 7% water by weight as determined using a Speed Vac Concentrator centrifuge (Savant Corp.) with the application of both vacuum and heating ($\sim 40^\circ\text{C}$) for 4 h. The samples were weighed before and after desiccation.

2.4. Viability testing

To ascertain the number of living organisms in a nanofibre sample, a piece of the fibrous material was weighed. LB medium (1.0 ml) was added to this piece and it was then held for 60 min at room temperature. The polymeric fibres dissolve completely under these conditions. The cells or viruses were dispersed by agitating the solution with a Vortex mixer. The sample was then diluted in LB and assayed for bacterial cells capable of forming colonies on LB plates containing 15 g of agar per litre or for bacteriophage particles as determined by plaque assay. The plaque assay was performed by mixing a dilution of the phage suspension with 0.1 ml of an overnight culture of W3110, adding 4 ml of molten top agar and

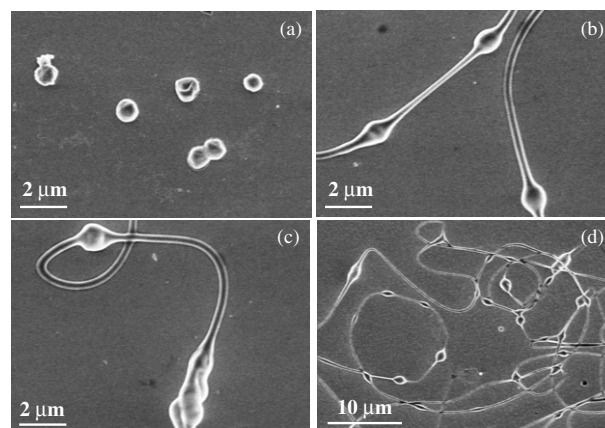


Figure 3. HRSEM micrographs of (a) individual *S. albus* cells, and ((b)–(d)) embedded *S. albus* cells in electrospun PVA nanofibres. (c) Shows the embedding of what looks to be an aggregate of several bacterial cells. (d) A lower magnification of these fibres.

pouring this over agar plates. The plates and top layer agar contained 10 g tryptone and 5 g NaCl per litre of H₂O; the plates contained 10 g agar while the top layer agar had 7 g. Incubations were at 37°C .

2.5. Microscopy

The specimens for high resolution scanning electron microscopy (HRSEM) and fluorescence microscopy were prepared by direct deposition of the electrospun nanofibres onto pieces of silicon wafer which were attached to the collector-disc edge. The micrographs were obtained by a secondary scattered-electron detector using a Leo Gemini 982 HRSEM at an acceleration voltage of 2–4 kV and a sample to detector distance of 2–4 mm. Visual inspection of samples of *E. coli* containing a red fluorescent protein were performed using a Leica inverted fluorescence microscope (DMIRE 2).

The specimens for transmission electron microscopy (TEM) analysis were prepared by direct deposition of the electrospun nanofibres onto a copper grid coated by a holey carbon film. The grids were attached to the collector-disc edge. The samples were examined using low electron-dose imaging and an acceleration voltage of 120 kV with a Philips CM120 TEM. Images were recorded with a Gatan MultiScan 791 CCD camera, using the Gatan Digital Micrograph 3.1 software package. For TEM analysis of bacteriophage T4, the phage were negatively stained using 2% uranyl acetate. A carbon-coated grid was placed on a 10 μl sample drop of T4 for 2 min, blotted with filter paper, stained with 2% uranyl acetate for 2 min, blotted again, and then air dried.

3. Results and discussion

The electrospun nanofibres had a diameter ranging between 250 and 400 nm and had a generally uniform thickness along the nanofibre without the formation of beads (figure 1(b)). HRSEM micrographs of embedded *S. albus* cells in electrospun PVA nanofibres are shown in figure 3. The *S. albus* cells are distributed along the as-spun nanofibres and the average distance between them is 6 ± 2 μm. In some

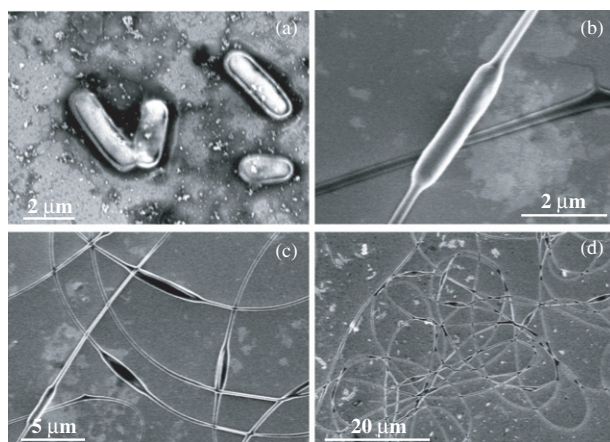


Figure 4. HRSEM micrographs of (a) individual *E. coli* cells, and (b)–(d) embedded *E. coli* cells in electrospun PVA nanofibres. (d) A lower magnification of these fibres.

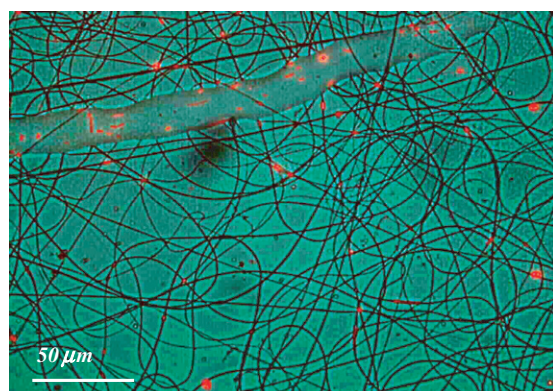


Figure 5. An image of fluorescent *E. coli* cells (the red spots) embedded in electrospun PVA-polymer nanofibres. Both a large fibre and individual nanofibres with embedded fluorescent cells are shown.

places an aggregation of cells within the nanofibres is observed (see figure 3(c)). Such aggregates were also observed before spinning and it is apparent that the electrospinning process has not disrupted these aggregates. In figure 4 HRSEM micrographs of *E. coli* cells are presented. It is clearly seen that the polymeric matrix entirely enclosed the embedded *E. coli* forming a local widening of the fibre. The cells are aligned longitudinally with the nanofibre axis. The average distance between the cell centres is $10 \pm 3 \mu\text{m}$. Additional support for the incorporation of the *E. coli* was achieved using fluorescent microscopy of fibres containing an *E. coli* strain that synthesizes a fluorescent protein as described above (see figure 5). This intracellular protein permits the detection of the embedded bacteria *in situ*. A thick fibre ($>10 \mu\text{m}$) is also present among the nanofibres of figure 5. These exceptionally thick nanofibres are apparently produced when bending instability does not take place, namely at the start and end of the electrospinning process in which a straight, thick compound jet is deposited on the grounded collector disc. TEM micrographs of embedded bacterial viruses T4 are shown in figure 6. In figure 6(a), three viruses stained by uranyl

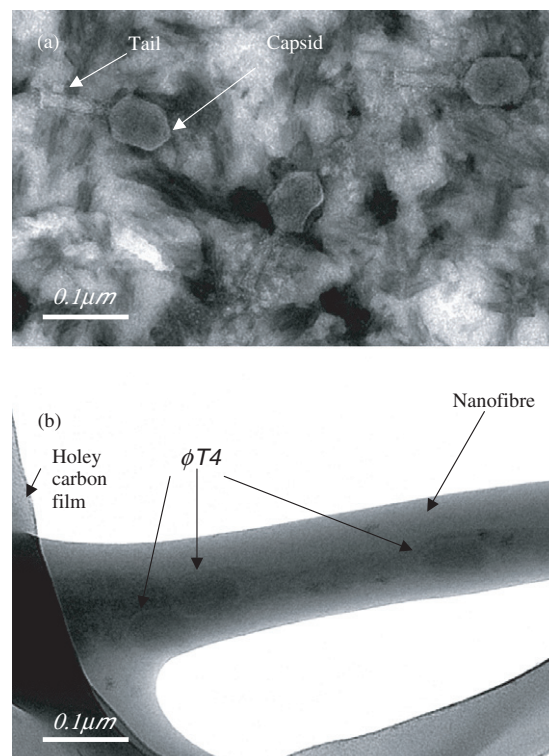


Figure 6. TEM micrographs of (a) stained T4 virus (the background is due to excess uranyl acetate that tends to aggregate), and (b) unstained T4 embedded in a PVA electrospun nanofibre.

Table 1. Viability of electrospun bacteria and bacteriophage. The numbers represent the relative viability (viability after spinning/viability before spinning). All organisms were suspended in LB before spinning. The sources of error are the weighing of the spun material, the dilution steps and the plating error. The total error is estimated to be between 20 and 40%.

	<i>E. coli</i>	<i>S. albus</i>	T4	T7	Lambda
Viability%	19	100	1	2	6

acetate are shown. The typical structure of a capsid and a tail can be clearly observed. The capsid width is about 85 nm and its length about 110 nm. The tail length is about 130 nm and its width about 20 nm. In figure 6(b), three viruses encapsulated inside a PVA nanofibre with a diameter of 160 nm are shown. Due to the relative low contrast between the polymer matrix and the unstained virus particles, the relatively narrow tail cannot be seen, although the capsid is clearly observed.

To study the viability of the bacteria and bacteriophages before and after spinning, their ability to form colonies on agar plates (bacteria) or to form plaques on host bacteria (bacteriophages) was ascertained at each step and then at various times after spinning. Exposure to PVA had little or no effect on the viability of the two bacterial species and the three types of bacteriophages tested here, even when these organisms remain in this solution for several days before assaying them. Immediately after electrospinning, the viability was found to be: *E. coli*, 19% (LB grown); *S. albus*, 100%; T4, 1%; T7, 2%; and λ , 6% (table 1). Both the Gram positive *S. albus* and the Gram negative *E. coli* have strong cell walls and can withstand at least $50\,000\times$ the force of

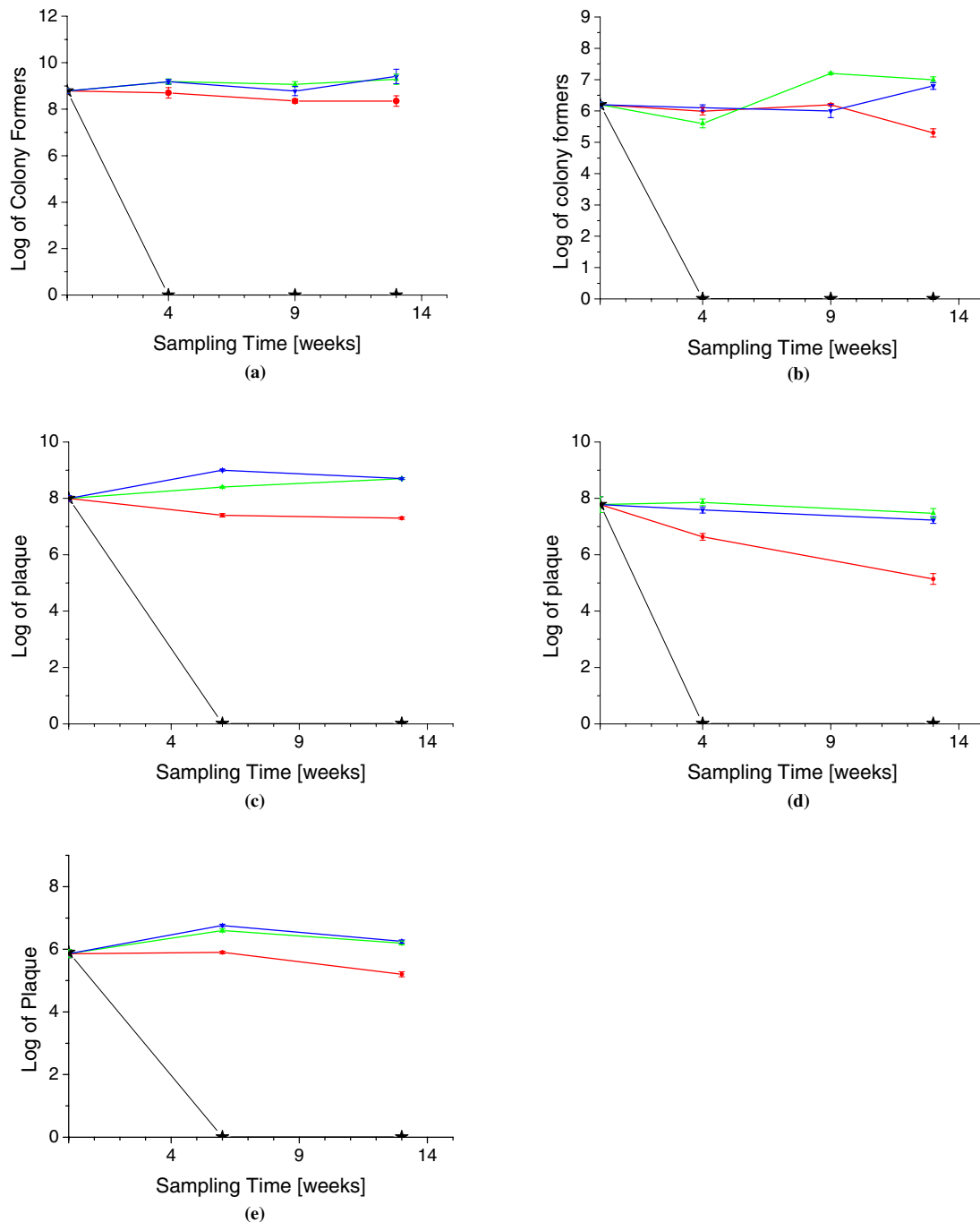


Figure 7. A semilog plot of the number of colony or plaque forming units per milligram of electrospun nanofibres versus sampling time in four temperatures (time = 0: immediately after the electrospinning process): ★ 24 °C; ● 4 °C; ▼ -20 °C and ▲ -55 °C. (a) *S. albus* cells, (b) *E. coli* cells, (c) T4, (d) T7 and (e) λ. The titres given represent an average of two or more plates per point.

gravity in high speed centrifuges without effect. Experiments were carried out with *E. coli* to determine whether survival during electrospinning could be improved. Cells grown in Vogel–Bonner minimal medium were much more susceptible to death during the electrospinning process than those grown overnight in LB. Cells grown in LB but harvested in the logarithmic phase of growth or grown in LB with continuous shaking for 5 days survive less well than those grown overnight in LB. A five-day-old culture was examined because *E. coli* is known to become more resistant to physical stress during

cessation of growth [29]. Cultures of *E. coli* grown in Vogel–Bonner medium and then washed with 10% glucose, sucrose or glycerol (table 2) and suspended with the same sugar were also examined but only glycerol gave a substantial increase in viability when the cells were subjected to electrospinning. Therefore, overnight cultures of *E. coli* were grown in LB and then centrifuged and washed with 5% and 10% glycerol. These were suspended with the same solution in which they had been washed. In 5% and 10% glycerol, viability was 48% and 22%, respectively (see table 2). Glycerol enters *E. coli* by facilitated

Table 2. Viability of *E. coli* suspended in different solutions. The bacteria were placed in different solutions before spinning and viability was assessed directly after spinning and compared to that before electrospinning. The numbers represent the relative viability. The sources of error are the weighing of the spun material, the dilution steps and the plating error. The total error is estimated to be between 20 and 40%.

	5% glycerol	10% glycerol	10% sucrose	10% glucose
Viability%	48	22	0.2	0.07

diffusion without chemical modification [30] and may protect the cells from the rapid dehydration that is expected to occur as the nanofibres are generated which may be the reason for the relatively low viability of *E. coli*. The evaporation of the solvent from electrospun fibres should be of the order of 10 ms, (see [7]). Since the mechanical stresses during electrospinning are about $5 \times 10^4 \text{ g cm}^{-1} \text{ s}^{-2}$ and these are far below those which *E. coli* can withstand ($3 \times 10^6 \text{ g cm}^{-1} \text{ s}^{-2}$ [31]), this species should easily survive electrospinning. Therefore, it seems that it is the rapid evaporation of the solvent rather than pressure that leads to cell death.

The bacteriophages studied here have an architecture that should make them susceptible to damage during electrospinning. While their capsids are expected to be quite resistant to physical forces (all can be subjected to forces in excess of 100 000–200 000 $\times$ force of gravity), their tails and especially their tail fibres are known to be sensitive to shearing forces. Since both λ and T4 have been assembled *in vitro*, it may be possible to differentiate between damage to the capsid and damage to the tail and tail fibres during electrospinning, but this has not been examined.

After the organisms were embedded in fibres, they were stored at room temperature, 4, -20 or -55°C and the viability of the stored material was periodically examined. As shown in figures 7(a) and (b), both bacterial species showed a complete loss of viability after one month at room temperature, some loss at 4°C during 3 months (*S. albus*, figure 7(a)) and 4 months (*E. coli*, figure 7(b)) but were essentially completely stable at -20 and -55°C . Similar results were found for all three bacteriophages studied here (see figures 7(c)–(e)). From these results it is probable that many organisms can be stored conveniently and efficiently at the two lower temperatures in this dry form.

4. Summary

In the present work it was found that viruses and bacteria can be encapsulated by electrospinning and that they managed to survive in spite of the pressure buildup in the core of the fibre and the electrostatic field during this process. Previously, neither viruses with a complex morphology nor whole bacterial cells have been successfully encapsulated by electrospinning. The present work shows that a range of organisms can be efficiently encapsulated. Several per cent of the bacteriophages, T4, T7 and λ , remain viable after spinning which makes this method attractive for phage therapy and for their use as viral vectors. *Staphylococcus albus* remains completely viable while *Escherichia coli* cells show a reduction in their colony forming ability to 19%,

which however can be improved to about 50% when they are suspended in 5% glycerol prior to spinning. After encapsulation, all organisms retain their viability for at least 3 months without further loss at -20 and -55°C . Therefore, this technique may represent an excellent alternative to lyophilization for the preservation of organisms for strain collections, for maintaining genetically modified bacterial strains of industrial importance, and for applications such as biosensing.

Moreover, electrospinning provides an excellent method for encapsulating and orienting biological materials (DNA, proteins, drugs, etc) and organisms. Electrospun nanofibre mats can be used to conveniently cover three-dimensional surfaces (e.g. tissues and organs) and release their contents for the potential treatment of wounds and cutaneous fungal infections [18, 21, 32, 33] and possibly for gene and phage therapy.

Acknowledgments

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